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Metabolic effects of short-term caloric restriction in mice with reduced insulin gene dosage

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2. Abstract

Caloric restriction (CR) is the only environmental intervention with robust evidence that it extends lifespan and delays the symptoms of ageing, but its mechanisms are incompletely understood. Based on the prolonged longevity of knockout models, it was hypothesized that the insulin-IGF pathway could be a target for developing a CR mimic. This study aimed to test whether CR has additive effects on glucose homeostasis and beta-cell function in mice with reduced insulin gene dosage. To study models with a range of basal insulin levels, wildtype C57BL/6J and mice on an *Ins2*$^{-/-}$ background, were put on 8 weeks of 40% CR. Both male and female mice rapidly lost weight due to a reduced WAT mass. Interestingly, absolute BAT mass was increased in female *Ins2*$^{-/-}$ mice, suggesting the possibility of increased thermogenic capacity. Glucose tolerance was improved and fasting glucose levels were reduced by CR in both wildtype and 45 and 70 week-old *Ins2*$^{-/-}$ mice. The effects of CR and reduced insulin on glucose tolerance were non-additive in 20 week-old mice. Interestingly, mice on CR generally exhibited an inability to further depress blood glucose after insulin injection, pointing to possible alterations in insulin sensitivity. In conclusion, our results demonstrate that CR can cause weight loss in the context of reduced insulin production, but that CR-improved glucose homeostasis does not occur near the 'insulin floor' in young mice. Collectively, these data shed further light on the relationships between caloric restriction, insulin and glucose homeostasis.

3. Introduction

As the median human lifespan rises with every generation, increasing our understanding of ageing becomes more important. Despite significant research efforts to uncover approaches that delay the onset of age-related diseases, caloric restriction (CR) is still the only environmental intervention that can extend lifespan and improve health of most species (Fontana & Partridge 2015; Kapahi *et al.* 2017). Nearly 80 years following the first CR study, many theories have been proposed to be the underlying mechanism of caloric restriction (McCay *et al.* 1939; Masoro 2009). Among these mechanisms, a subset of these hypotheses involve hormonal and endocrine signaling, with important roles for elements of the nutrient-sensing pathway activity (Masoro 2005, 2009; Fontana *et al.* 2010a). Similarly, experiments have extended longevity in mice and lower model organisms with alterations in GH or IGF1/insulin signaling (Tatar *et al.* 2003; Mair & Dillin 2008; Fontana & Partridge 2015). The characteristic profile of CR treated animals, such as reduced body weight, reduced GH and IGF1 levels, decreased plasma levels of insulin and glucose, reduced fertility, and delayed puberty, are often found in animals with repression in the activity of the GH-IGF1 axis (Berryman *et al.* 2008; Anisimov & Bartke 2013). Based on these similarities, it is feasible that CR and reductions in the GH/IGF1 axis may increase lifespan through similar processes (Berryman *et al.* 2008). Various studies have looked whether CR has additive effects on lifespan in mice with mutations in the GH-IGF1 axis, but results remain inconclusive (Bartke *et al.* 2001; Masternak *et al.* 2004; Bonkowski *et al.* 2006; Gesing *et al.* 2014; Wiesenborn *et al.* 2017).

CR interventions often result in reduced body weight and adiposity, suggesting a possible link with longevity. Indeed, it has been proposed that adipocytes modulate the pace of ageing by secreting peptides like TNFα, adiponectin, leptin or angiotensin, that seem to promote ageing when present in excess (Barzilai & Gupta 1999; Barzilai & Gabriely 2001; Okita *et al.* 2012). Previous research has shown that short term CR reduces adipokine levels, improves insulin/IGF1 signaling, and reduces reproductive investment (Mitchell *et al.* 2015). Notably, fat-specific knockout of the insulin receptor has been reported to extend mouse lifespan by reducing adiposity with altered secretion of adipokines, including higher adiponectin and lower pro-inflammatory cytokines (Blüher *et al.* 2002, 2003). The complex effects of deleting insulin receptors in a single tissue, as well as caveats related to the reduction of IGF1 signaling

through hybrid receptors (Belfiore *et al.* 2009), leave the role of insulin itself on metabolism and longevity ambiguous. Thus, the relationship between insulin and acute CR remains unclear.

One way to modify insulin signaling *in vivo* is the direct modulation of insulin gene dosage using combinations of null alleles (Mehran *et al.* 2012; Templeman *et al.* 2015, 2016; Dionne *et al.* 2016) For example, we have used mice that lack the ancestral *Ins2*$^{-/-}$ gene, but compensate to maintain baseline levels of insulin secretion and approximately normal glucose homeostasis via the mouse specific *Ins1* gene (Duvillié *et al.* 1997; Leroux *et al.* 2000). When challenged with a high fat diet, we have previously shown that these animals, which are genetically incapable of sustained hyperinsulinemia, exhibit long-term protection against diet-induced-obesity and eventually improved insulin sensitivity and prolonged lifespan (Mehran *et al.* 2012; Templeman *et al.* 2015, 2016, 2017). To investigate the relationship of insulin and the effects of CR, we have combined insulin gene manipulation with CR. In a recent study, we found that long-term caloric restriction reduced islet insulin content to the same extent as the removal of one *Ins1* allele, and that the combination of CR and *Ins1* heterozygosity were non-additive (Dionne *et al.* 2016). Interestingly, while CR improved glucose homeostasis in *Ins1*$^{+/+}$ and *Ins1*$^{+/-}$ mice, both sets of CR mice exhibited a paradoxical increase in age-associated adiposity in the absence of the *Ins2* gene, despite lower overall body weight (Dionne *et al.* 2016). It remained unclear whether the increase in relative adiposity was due to the chronic nature of the CR employed or whether this was a direct effect observable after short-term CR.

In the present study, we employed a short-term CR intervention in normal mice and in mice with low insulin. We studied wildtype mice, as well as both male and female *Ins2*$^{-/-}$ mice at different ages, after a short-term CR intervention. The outcomes of short-term CR depended on the context of normal or reduced insulin gene dosage, as well as differences between male and female *Ins2*$^{-/-}$ mice.

4. Methods

4.1 Experimental Animals

The *Ins2* and *Ins1* mutant mice were created at INSERM by J. Jami and colleagues (Duvillié *et al.* 1997) and bred further to obtain *Ins1*$^{+/+}$*Ins2*$^{-/-}$ or *Ins1*$^{+/-}$*Ins2*$^{-/-}$ littermates with a mixed background

(predominately C57BL/6J and 129 strains)(Templeman *et al.* 2017). Mice were housed in specific pathogen free conditions on ventilated (50 air changes per hour), autowater Ehret mouse cage racks at ambient room temperature of 21^{o}C. Animals were separated and housed individually a week prior to the start of the experiment, animals in the AL and CR group were matched based on body weight. Control animals that did not participate in the physiological experiments were group-housed until sacrifice. Male C57BL/6J (The Jackson Laboratory, USA) were used as a wildtype reference strain, but are not direct controls for the other studies.

Mice were fed LM-485 chow (Teklad Diet Madison, WI), either *ad libitum* or calorie restricted. Caloric restriction was defined as 60% of average food intake of both genotypes from age-matched mice from an earlier cohort. Food was provided using an automated feeder (F14 Aquarium Fish Feeder, Fish Mate) that would drop weighted food pellets in three quasi-equal "meals" during the dark phase. The meals were dispensed two, five and eight hours after initiation of dark phase of the 12-hour light/dark cycle.

Animals were placed on 8-10 weeks of caloric restriction, starting at different life stages; adolescent mice (20w old), adult mice (45w old) and aged mice (60-70w old). In order to allow adaptation to restricted feeding, the animals were fed one week with 90% food intake and a second week with 75% food intake and 60% food intake thereafter. Body weight was measured twice a week and animals were sacrificed after 8-10 weeks. The night before sacrifice the meal times of the CR mice were shifted forward by 4 or 5 hours so that mice were terminated within 1 hour after feeding. All animal procedures were approved by the University of British Columbia Animal Care Committee, in accordance with the guidelines set out by the Canadian Council for Animal Care.

4.2 Physiological experiments

Two weeks before and after the CR treatment, blood was collected for analysis, blood glucose response to intraperitoneal administrations of glucose (2g/kg)(GTT) or an insulin analog (0.75U/kg of Humalog; Eli Lilly, Indianapolis, IN, USA)(ITT) was followed for 2 hours using OneTouch Ultra2 glucose meters (LifeScan Canada Ltd, Burnaby, BC, Canada). A dose of 2g/kg glucose was sufficient to induce

glucose stimulated insulin secretion, and we found that a dose of 0.75U/kg insulin most optimal to test insulin sensitivity. Higher doses of insulin were tested in pilot insulin tolerance test experiments but caused several of the mice to go into life-threatening hypoglycaemia, requiring rescue with exogenous glucose (data not shown). Intraperitoneal glucose-stimulated (2g/kg) insulin secretion (GSIS) was measured after mice were fasted for 4 hours, initiated within 2 hours after light turned on, to provide a postprandial state for glucose homeostasis measurements and blood sampling. Meal times of the CR mice were shifted forward by 4 or 5 hours so that their final meal was given 1 hour prior to fasting initiation. During the GSIS, blood from the saphenous vein was collected 3 times over 30 minutes. Plasma insulin levels were determined by mouse ultrasensitive insulin ELISA kit (Alpco Diagnostics, Salem, NH, USA), according to manufacturer's instructions. Baseline subtracted area under the curve of both glucose and insulin, in IPGTT and GSIS studies, respectively, was calculated. Similarly, area over the curve was calculated starting at baseline glucose levels in the ITT experiments.

4.3 Islet isolation and insulin content determination

Islet isolation was performed using a 3G needle to inject 2 mL Liberase TL (1000 units/mL dissolved in 25 mL Hanks buffer containing 157 mM NaCl, 5.4 mM KCl, 4.2 mM NaH2PO4, 4.1 mM KH2PO4, 10 mM HEPES, 1 mM MgCl2 and 5 mM glucose (Salvalaggio *et al.* 2002). Incubation occurred for 7 min at 37C to fully digest exocrine material before filtering the suspension with a 70 um Nylon Falcon Cell Strainer. Islets were handpicked and cultured in RPMI medium (containing 100U/ml penicillin, 100 µg/ml streptomycin, 10% FCS, pH of 7.4) at 37°C and 5% CO2 at saturated humidity overnight. Islet perifusions were performed as described (Dror *et al.* 2007) using 150 size-matched islets per group. Islets were equilibrated under basal (3 mM glucose) conditions and stimulated with either 15 mM glucose and 30 mM KCL conditions.

A total of 10 medium sized islets were lysed in acid-ethanol buffer to determine insulin content. Sonicated samples were diluted to linear range of the mouse ultrasensitive insulin ELISA kit (Alpco Diagnostics, Salem, NH, USA) and insulin concentration determined according to manufacturer's instructions.

4.4 Tissue analysis

At termination, gonadal (gWAT), subcutaneous (scWAT) and mesenteric (mWAT) fat were dissected, as well was interscapular brown adipose tissue (BAT). Tissues were weighed and flash-frozen in liquid nitrogen before being stored at −80°C or fixed in 4% paraformaldehyde for 24h, embedded in paraffin and cut into 5 µm thick sections that were stained by hematoxylin and eosin. Images were taken with identical exposure settings and time with Zeiss Axio Imager A1. Adipocyte size assesment was done by randomly measuring 100 adipocytes each mouse, using ImageJ.

4.5 Real time qPCR

RNA was isolated from mouse fat tissues or ±100 islets using the RNEasy mini Kit (Qiagen, Mississauga, ON, Canada) with 1% β-mercaptoethanol for islets, or phenol–chloroform extraction for adipose tissue with TRIzol (Invitrogen). Transcript levels of synthesized cDNA (Quanta Biosciences, Gaithersburg, MD) were measured with SYBR green chemistry on a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Ct-values were normalized by a standard curve and presented as relative expression of beta-actin (islets) or TBP (fat). qPCR primers are listed in Supplementary table 1.

4.6 Statistical analysis

Data presented are mean ± standard error of the mean (SEM), and analysed by ANOVA followed by Tukey or Bonferroni *t*-tests, or by unpaired Student's *t*-tests. Gene expression analysis was performed by multiple t-tests between the *ad libitum* fed group and the CR group within each adipose depot. Significance was achieved when $p \leq 0.05$ using GraphPad Prism 6.0.

5. Results

5.1 Effects of CR on insulin homeostasis and whole body fat storage

CR has been reported to improve metabolic healthspan and delay ageing (Fontana *et al.* 2010a), with decreased leptin, IGF1 and insulin levels, as well as improved glucose homeostasis, within 3 months

178 (Mitchell *et al.* 2015). In contrast to results from a previous short-term CR study (Mitchell *et al.* 2015), in
179 this study of short-term CR, 60 week-old male C57BL/6J mice showed no reduction in insulin
180 homeostasis, shown by insulin content (Fig. 1A), *ex vivo* perifusion of islolated islets (Fig. 1B) and fasting
181 insulin levels (Fig. 1C). However, it did significantly improve glucose homeostasis, seen in a reduction of
182 fasted glucose levels (Fig. 1D) and improved glucose tolerance measured after injections of 2g/kg glucose
183 (Fig. 1E). This improved glucose tolerance occurred despite a lack of observable differences in glucose-
184 stimulated insulin secretion under the conditions we used (Fig. 1F). All mice with reduced glucose levels
185 due to CR responded without the typical drop in glucose after an exogenous insulin injection of 0.75U/kg
186 (Fig. 1G). Collectively, these results suggested that despite no observed changes in the insulin secretion
187 profile of CR-fed wildtype animals, their glucose tolerance is still improved.

188 We next conducted a thorough analysis of adipose tissue size, cellular morphology and gene
189 expression in these *ad libitum*-fed or CR aged male C57BL/6J mice. Ten weeks of CR in these mice
190 resulted in a significant reduction body weight (Fig. 1H), reflected in a reduced liver size and gonadal fat
191 weight. Other major organs such as heart and kidney were not affected by the diet treatment (Fig. 1I).
192 Histochemical analysis revealed no significant differences in adipocyte size within all different fat pads
193 (representative pictures in Fig. 1J and quantitative adipocyte size assessment of scWAT, gWAT and
194 mWAT, Fig. 1K,L,M respectively). The recently proposed hypothesis that CR promotes the development
195 of functional beige fat (Fabbiano *et al.* 2016) was not confirmed within these aged male C57Bl-6J mice in
196 either gWAT nor scWAT assessed mRNA markers of thermogenesis. If anything, there was a consistent
197 trend towards a decrease in the expression of *Ucp1*, a mitochondrial membrane protein that contributes
198 to non-shivering thermogenesis (Fig. 1N) (Ricquier & Bouillaud 2000). Interestingly, *Insr* expression of
199 scWAT was significantly increased after a CR diet, suggesting the possibility of increased insulin
200 sensitivity of this adipose depot. Interestingly, BAT size was not changed after CR, but major changes
201 were seen in the gene expression profile (Fig. 1N). Expression of *Ucp1, Cox4* and *Prdm16*, genes involved
202 in thermogenesis, tended to be decreased, as well as genes involved in lipogenesis and adipogenesis
203 such as *Pparg, Fas* and *Acaca* (Fig. 1N). Therefore, based on our gene expression data with these
204 C57BL/6J mice, we cannot attribute the beneficial effects of CR on energy homeostasis to increased

thermogenic activation of either BAT or WAT. Collectively, these experiments suggest that even though whole-body insulin sensitivity does not appear improved by ITT in these CR C57BL/6J mice, individual tissues can still likely have altered responses to insulin.

5.2 Metabolic effects of CR in mice with reduced insulin dosage

In order to determine whether CR and reduced insulin signalling have additive effects on glucose lowering, we next used male mice with an *Ins2*$^{-/-}$ background. As expected, mice lacking the *Ins2* gene have ~70% less islet insulin content compared to wildtype C57BL/6J animals (Fig. 2A). Despite their lower insulin reservoir, we have shown previously that these animals are healthy without significant impairment of glucose-insulin homeostasis (Leroux *et al.* 2000; Mehran *et al.* 2012; Templeman *et al.* 2015, 2016; Dionne *et al.* 2016). In the present study, we studied *Ins2*$^{-/-}$ mice with and without 10 weeks of CR. In these mice, CR did not decrease insulin content in *Ins2*$^{-/-}$ CR mice (Fig. 2A), in agreement with our previous investigation of lifelong CR wherein islet insulin content and beta-cell area were also not further reduced in female mice with 2 or 3 insulin alleles inactivated, at any age tested (Dionne *et al.* 2016). In this study, fasting insulin levels seemed lower in CR *Ins2*$^{-/-}$ mice, but the variability and bimodal distribution in the *ad lib*-fed mice meant that differences were not significant with this relatively low sample size (Fig. 2B). Nevertheless, in the present study, as in our previous work, CR reduced lowered fasting glucose levels (Fig. 2C) and improved glucose tolerance despite secreting lower circulating insulin compared to the *ad libitum*-fed animals (Fig. 2D,E), suggesting that glucose uptake was increased independently of insulin alterations. Indeed, insulin tolerance tests, using exogenous insulin injection of 0.75U/kg, revealed a flat pattern of lowered fasting glucose that was maintained for 120 minutes without the typical drop in glucose (Fig. 2F). This flat pattern is reminiscent of partial insulin resistance. Collectively, the findings that aged *Ins2*$^{-/-}$ mice exhibit improved glucose tolerance were consistent with our observations of mice with a pure C57Bl/6 background. However, this improved glucose tolerance is not associated with improved whole-body insulin sensitivity under the conditions we tested.

5.3 Histological and gene expression changes in WAT after CR

We next investigated fat pad characteristics and marker genes involved in adipose energy homeostasis in male *Ins2*$^{-/-}$ mice. As expected, body weight dropped after 10 weeks of CR (Fig. 2G), reflected in significantly smaller gonadal and mesenteric fat pads and without affecting other organs like liver, heart and kidney (Fig. 2H). BAT size was unchanged after CR, however genes involved in thermogenesis, like *Ucp1* and *Prdm16*, were significantly downregulated. Decreased expression levels of lipogenic genes, like *Fasn* and *Acaca*, *Ppargc1a* (a coregulator of mitochondrial biogenesis) and apoptosis marker *Ddit3* were also observed in the BAT of CR mice (Fig. 2I). *Cidec* expression was significantly increased in BAT of *Ins2*$^{-/-}$ mice on a CR diet (Fig. 2I), in agreement with previous findings of Matsusue et al. (Matsusue 2010). Analysis of three different white adipose fat tissues at the levels of mRNA expression showed increased expression of *Fasn* and *Acaca*, genes that are both involved in lipogenesis. We also observed significantly increased levels of both *Ppargc1a* and *Cidea* gWAT (Fig. 2I), consistent with fat storage (Abreu-Vieira *et al.* 2015). Together, these data suggests that CR leads to an adaptation of WAT size and gene expression.

5.4 CR improves glucose homeostasis in female mice, independent of age or insulin dosage

Sex-specific regulation of glucose metabolism, insulin resistance and energy expenditure has been previously reported (Widdowson 1976; Valle *et al.* 2005; Varlamov *et al.* 2014; Mauvais-Jarvis 2015). Thus, we next assessed the effects of CR on female with low insulin gene dosage at 20 weeks, 45 weeks, and 70 weeks of age after 8 weeks of CR. We set out to test the hypothesis that short-term CR is still beneficial for energy metabolism during different life stages and might have different metabolic effects when comparing mice lacking 2 alleles versus mice lacking 3 insulin alleles. However, we did not observe statistically significant differences between *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ and *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice in any of our tests (Fig. S1). Therefore, the data were pooled to provide extra power to resolve the effects of CR in the context of low insulin (relative to C57BL/6J wildtype mice). In all three age groups, body weight dropped significantly (Fig. 3A). Compared to their original weight, the weight loss in the oldest females was slightly greater compared to the males at the same life stage. Islets isolated from these mice had significantly reduced insulin content at all ages tested (Fig. 3B), in contrast to what we observed in the

males (Fig. 1A,2A). We did not observe statistically significant differences in fasting insulin levels between CR mice and their controls (Fig. 3C), although mice on CR showed decreased fasting glucose levels at all ages tested (Fig. 3D). CR mice released less insulin in response to a glucose bolus at 20 weeks and 45 weeks of age, but not at 70 weeks of age (Fig. 3E). Interestingly, these CR mice had improved glucose tolerance at 45 and 70 weeks of age (Fig. 3F). At 20 weeks, the CR-induced improvement in glucose tolerance was not observed, demonstrating non-additivity in these young female mice with reduced insulin gene dosage (Fig. 3F). Insulin tolerance tests also revealed a similar pattern of apparent insulin resistance in the CR mice to what was observed in the males (Fig. 3G). Thus, our data demonstrate that in the context of reduced insulin gene dosage, short-term CR improves glucose homeostasis associated with paradoxical reduction in insulin secretion and insulin sensitivity. These phenomena are relatively consistent through the age range we tested.

5.5 Effects of CR on gene expression and depot size in BAT and WAT

In contrast to our findings in males, 45 week-old female mice showed more pronounced changes in WAT and BAT gene expression. All white fat depots measured (gonadal, subcutaneous and mesenteric) were significantly smaller (Fig. 4A,B), due to a decrease in adipocyte size (Fig. 4C,D,E,F). In the postprandial state, all fat depots of CR mice showed increased expression of lipogenic genes like *Fas* and *Acaca* consistent with the optimization of energy storage (Fig. 4G). Markers involved in browning of white adipose tissue were increased in the scWAT and mWAT depots, specifically *Adrb3, Ppargc1a, Prmd16, Cidea,* and *Cox4* expression. These genes would be predicted to stimulate *Ucp1* activity and thermogenesis (Okita *et al.* 2012; Abreu-Vieira *et al.* 2015; Garcia *et al.* 2016). However, differences in *Ucp1* expression were not significant (Fig. 4G). CR females also exhibited a slight increase in BAT mass, although this was also not statistically significant (Fig. 4A,B). Together, results from these female *Ins2*$^{-/-}$ mice suggest a shift between white and brown adipocyte gene expression profiles.

6. Discussion:

285 The aim of this study was to investigate the effects of short-term CR on glucose metabolism and
286 adipose tissue in the contexts of age and genetically lowered insulin. One of the most consistent findings
287 in animal models of CR is a significant improvement in glucose homeostasis (Colman *et al.* 2009; Fontana
288 *et al.* 2010b). Others have observed improvement in glucose tolerance, together with reduced levels of
289 leptin, insulin and IGF1, after only 3 months of CR (Mitchell *et al.* 2015). In the present study, we
290 confirmed the expected rapid improvement in glucose tolerance with CR, except in young female mice
291 with reduced insulin gene dosage.
292 The interpretation of the role of circulating insulin on the effects of CR is complicated by the fact
293 that fasting insulin levels were not dramatically different between the different strains of mice and at the
294 different ages tested, despite a significant reduction in insulin content in the mice with reduced insulin
295 gene dosage. Fasting insulin averaged 0.6-0.7 ng/ml in the male 60 week-old C57BL/6J mice regardless of
296 CR. Male *Ins2*$^{-/-}$ mice were able to compensate with increased insulin secretion such that fasting insulin
297 averaged 0.75 ng/ml with *ad libitum* feeding and 0.5 ng/ml with CR. This compensation for reduced
298 insulin gene dosage is consistent with previous studies, and we have found that this compensation is
299 more robust in male mice (Leroux *et al.* 2000; Templeman *et al.* 2016, 2017). Consistent with this, female
300 *Ins2*$^{-/-}$ mice exhibited the lowest fasting insulin levels of any group in our investigation, averaging 0.2
301 ng/ml regardless of CR status at 20 weeks of age and increasing to 0.4 ng/ml by 70 weeks of age.
302 Interestingly, it was only at the lowest circulating level in the 20 week-old female mice where CR did not
303 further improve glucose tolerance. This non-additive effect suggests that CR and extreme insulin
304 reduction act on glucose homeostasis via at least some common mechanisms in young mice. Other
305 effects of CR, including the expected weight loss were maintained at all ages, indicating that the
306 mechanisms controlling glucose homeostasis and weight loss in CR are distinct. The molecular
307 mechanisms underlying these differences require further study and are beyond the scope of this
308 investigation.
309 Our current study demonstrated beneficial effects of CR in aged mice, regardless of genotype. A
310 previous study had suggested that the age when CR is started, the severity of restriction, and the strain
311 or genetic background of the animals determines the magnitude of life extension (Fontana & Klein 2007).

312 Another study in mice demonstrated that initiating CR shortly after weaning (to age 6 months) caused a
313 proportionate 30% to 60% increase in maximum life span, whereas a reduction in calorie intake started
314 in adulthood extended maximum life span by only 10% to 20% (Weindruch & Walford 1982).

315 Furthermore, the mechanisms by which CR improves glucose homeostasis remain to be fully
316 elucidated. We did not observe differences in fasting insulin or glucose-stimulated insulin secretion in
317 our wildtype mice as shown previously (Mitchell *et al.* 2015), suggesting a metabolic improvement
318 independent of insulin secretion. The insulin tolerance tests changed the profile of blood glucose after a
319 bolus insulin injection. Differences in metabolic parameters between our study and others could possibly
320 be the result of our feeding protocol of 3 small meals during the dark phase which reduces the time of
321 fasting in the CR animals compared to standard protocol of 1 meal a day. In all cases, the significantly
322 lower fasting glucose complicates the interpretation of these data, and we do not have data on counter-
323 regulatory signals that are likely robust under these conditions. It should be also noted that the insulin
324 tolerance test is not ideal for a full assessment of insulin sensitivity. Additional studies to further
325 elucidate these phenomena will require hyperinsulinemic-euglycemic clamp experiments.

326 Our study identified possible sex differences in the metabolic response to short-term CR. We
327 documented the expected rapid decrease in body weight and blood glucose levels after CR treatment,
328 but found that it was more pronounced in females than in males. Some authors have argued that
329 females conserve their energy more efficiently and are more resistant to CR because of their relative
330 importance for reproduction and the survival of the species (Widdowson 1976; Valle *et al.* 2005).
331 However, in our study the female CR mice lose an equal percentage of their starting body weight. Female
332 mice showed a reduction in their islets insulin content due to CR, which wasn't found in males,
333 suggesting that female *Ins2*$^{-/-}$ mice may be more susceptible to the effects of CR. BAT activity was
334 diminished in male *Ins2*$^{-/-}$ mice, whereas this tissue was still active in the female *Ins2*$^{-/-}$ CR mice.
335 Furthermore, upregulation of genes involved in lipogenesis and thermogenesis in scWAT are more
336 pronounced in females. The molecular mechanisms underlying the sex differences in the metabolic
337 responses to CR require further study. In female mice with reduced insulin, CR reduced the size of white
338 adipocytes, which has been linked to alterations in altered adipokine secretion. Hypertrophic adipocytes,

possessing more triglycerides, have been proposed to secrete less adiponectin and more pro-inflammatory cytokines whereas small adipocytes are generally found to be more sensitive to insulin and act as a powerful buffer taking up free fatty acids during the postprandial period. For this reason, reducing adipocyte size by CR is considered beneficial for a healthy lifespan (Okita *et al.* 2012).

It has been proposed that the activation of BAT and the browning of WAT play important roles in the effects of CR. Originally, it was believed that WAT and BAT had distinct morphology and function, with WAT being a major source for triglyceride storage and adiponectin secretion to enhance insulin sensitivity, and BAT playing an important role in energy expenditure and thermogenesis (Saely *et al.* 2011; Okita *et al.* 2012). Lately, the distinction between BAT and WAT has become less rigid, with observations of browning of WAT (i.e. increased *Ucp1* expression) under certain circumstances (Puigserver & Spiegelman 2003), including CR (Fabbiano *et al.* 2016) and strongly reduced circulating insulin (Mehran *et al.* 2012). Similarly, we found reduced adipocyte size and increased expression of some thermogenic genes in female *Ins2*$^{-/-}$ mice with CR. We examined the expression levels of *Ucp1* mRNA, which is critical for thermogenic activity (Puigserver *et al.* 1998; Puigserver & Spiegelman 2003) and is known to be positively correlated with metabolic inefficiency in overfeeding (Nedergaard *et al.* 2001). We did not observe an increase in *Ucp1* mRNA, and in fact there were robust reductions under some conditions. Nevertheless, we observed tendencies for increases in genes known to control *Ucp1* expression and thermogenesis, such as *Ppargc1a*, *Nrf1* and *Cox4*. The molecular mechanisms mediating the possible functional remodeling of adipose tissue in the context of short term CR require further study. A previous study suggested that CR down-regulated the mitochondrial electron transport chain but enhanced fatty acid biosynthesis in BAT, suggesting that in CR animals BAT may change its function from an energy consuming system to an energy reservoir system (Okita *et al.* 2012). It was suggested that CR promotes fatty acid biosynthesis and the metabolic process involving pyruvate, citrate, oxaloacetate and malate in both WAT and BAT based on increased ACLY (ATP citrate lyase) phosphorylation and upregulated *Fasn* mRNA levels for de novo fatty acid biosynthesis (Okita *et al.* 2012). A previous study from our group also found histological evidence for 'whitening' of BAT after long term CR (Dionne *et al.* 2016). However, with the possible exception of upregulated expression of

lipogenic genes in female *Ins2*$^{-/-}$ mice, we did not find robust evidence for whitening of BAT with short term CR in the present study.

In conclusion, we found that short-term CR promotes metabolic processes that are favorable for glucose homeostasis in C57BL/6J and *Ins2*$^{-/-}$ mice. CR improves glucose tolerance, except in young mice with reduced insulin gene dosage, suggesting both insulin-independent and insulin-dependent mechanisms of CR that are age-, and to some degree, sex-dependent. The beneficial actions of CR are associated with both WAT and BAT remodeling towards increased thermogenesis. Understanding and harnessing mechanisms associated with CR may lead to additional ways to improve glucose and lipid homeostasis.

7. Declaration of interest

The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

8. Funding:

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9. Author Contributions:

M.B.D. lead the design, conduct and interpretation of experimental studies, and wrote the manuscript. D.A.D. and D.F.H assisted with design, conduct and interpretation of experimental studies, and edited the manuscript. J.K.K. co-supervised M.B.D. and edited the manuscript. J.D.J. co-supervised M.B.D., conceived the studies, assisted with interpretation of experimental studies, edited the manuscript and is the guarantor of this work.

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Figure legends

Figure 1: **Caloric restriction improves metabolic health in male C57BL/6J mice by decreasing body weight and fasting glucose levels**. The effects of CR vs. ad libitum feeding on pancreatic islets insulin content (A), ex vivo glucose-stimulated (2g/kg) insulin secretion (B), 4-hour fasting insulin levels (C), 4-hour fasting glucose levels (D), intraperitoneal glucose (2g/kg) tolerance test following 4-hour fast (E), intraperitoneal glucose-stimulated (2g/kg) insulin secretion following 4-hour fast (F), insulin (0.75U/kg) tolerance test following 4-hour fast (G), body weight (H) and composition (I); n = 7-10, together with fat composition (J)(representative pictures, magnified 100x scale bar = 200 μm) and quantitative adipocyte size assessment of scWAT (K), gWAT (L) and mWAT (M) of *ad libitum* and CR mice; n = 3, and altered mRNA expression within different fat depots (N); n = 7. Results are shown as mean ± SEM. Significance is indicated by *for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ between *ad libitum* vs. CR animals. Gene expression data was analyzed by multiple t-tests between the *ad libitum* fed group and the CR group within each adipose depot, with $p<0.05$ as considered significant.

Figure 2: **Caloric restriction improves glucose homeostasis in *Ins2*$^{-/-}$ mice independent of insulin content**. Reduced pancreatic islets insulin content in *Ins2*$^{-/-}$ mice compared to wildtype (A). The effects of CR on 4-hour fasting insulin levels (B), 4-hour fasting glucose levels (C), intraperitoneal glucose (2g/kg) tolerance test following 4-hour fast (D), intraperitoneal glucose-stimulated (2g/kg) insulin secretion following 4-hour fast (E), Insulin (0.75U/kg) tolerance test following 4-hour fast (F) in *Ins2*$^{-/-}$ mice by changes in body weight (G) composition (H); n=8-10. Altered mRNA expression within different fat depots (I); n = 8-10. Results are shown as mean ± SEM. Significance is indicated by *for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ between *ad libitum* vs. CR animals. Gene expression data was analyzed by multiple t-tests between the *ad libitum* fed group and the CR group within each adipose depot, with $p<0.05$ as considered significant.

Figure 3: **CR-induced metabolic improvements are independent of age in female *Ins2*$^{-/-}$ mice.** The effects of CR vs. ad libitum feeding on body weight (A), pancreatic islets insulin content (fed)(B), 4-hour

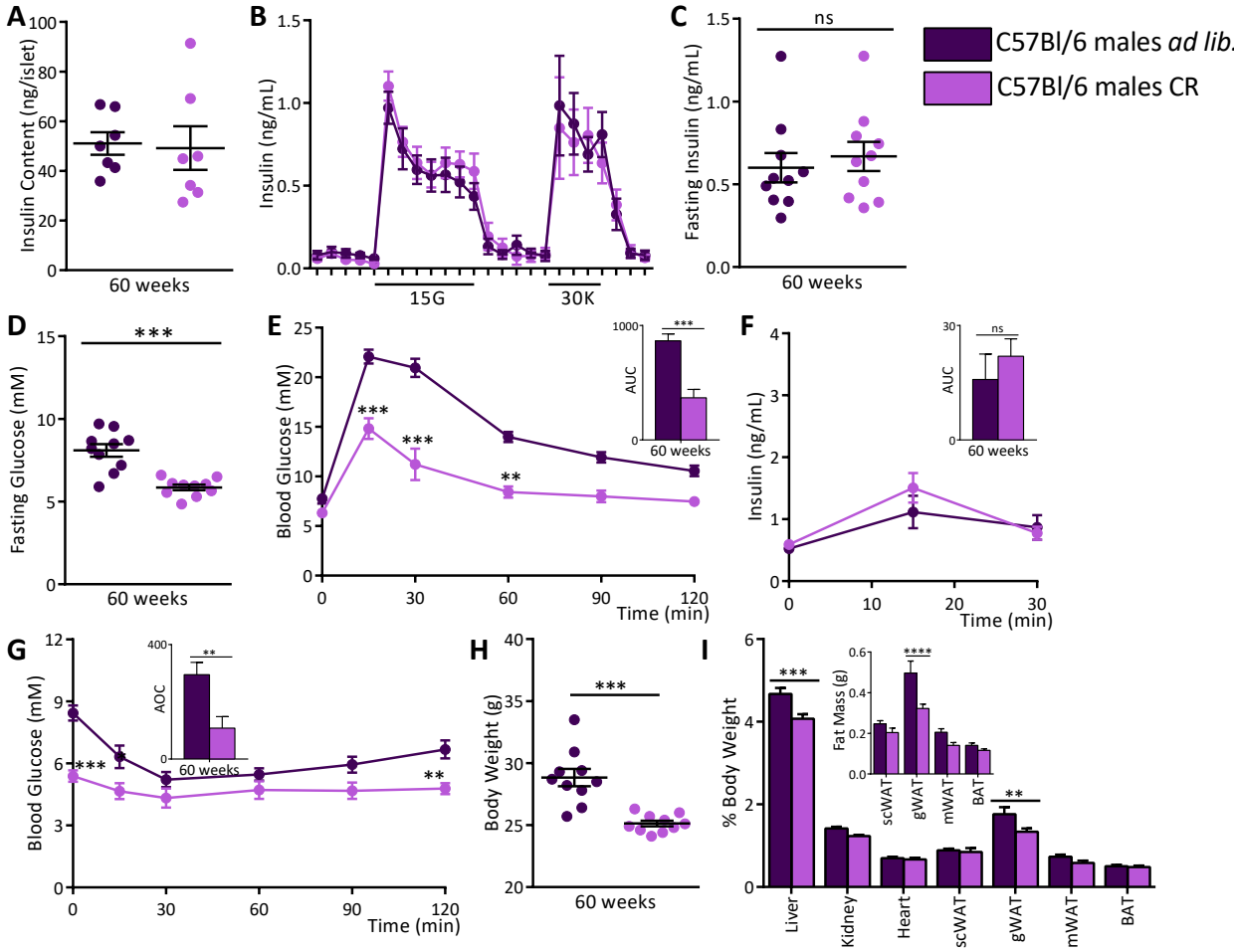
fasting insulin levels (C), 4-hour fasting glucose levels (D), intraperitoneal glucose-stimulated (2g/kg) insulin secretion following 4-hour fast (E), intraperitoneal glucose (2g/kg) tolerance test following 4-hour fast (F), and insulin (0.75U/kg) tolerance test following 4-hour fast (G) in female *Ins2*$^{-/-}$ mice (combined *Ins1*$^{+/-}$ and *Ins1*$^{+/+}$ genotypes) of different ages. Animals used; n=10-16 (20 weeks), n=18-20 (45 weeks) and n=8-10 (70 weeks). Results are shown as mean ± SEM. Significance is indicated by *for p<0.05, ** for p<0.01, *** for p<0.001 between *ad libitum* and CR animals. LOD, limit of detection.

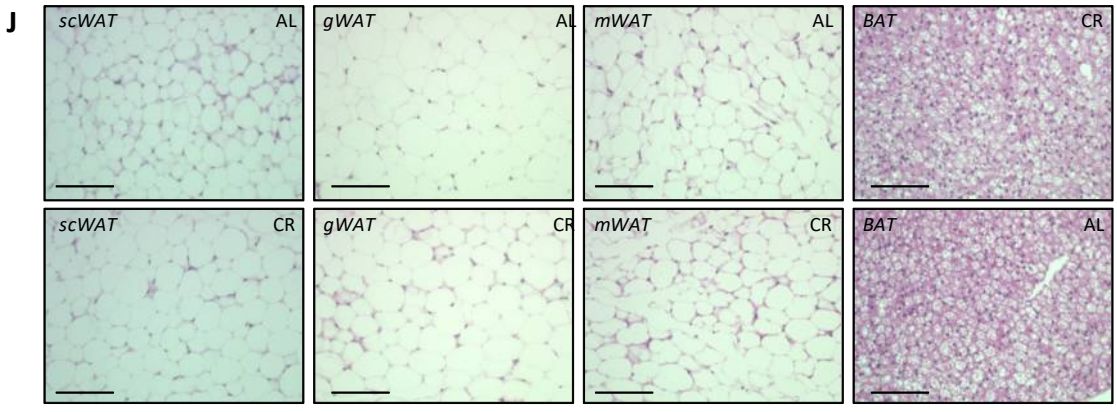
Figure 4: **CR-induced activation of BAT and browning of WAT in female *Ins2*$^{-/-}$ mice.** Body composition; n=13-21 (A/B), representative pictures of fat composition, magnified 100x scale bar = 200 μm (C), adipocyte size assessment of scWAT (D), gWAT (E), mWAT (F); n=5, and altered mRNA expression within different fat depots in female mice (combined *Ins1*$^{+/-}$ and *Ins1*$^{+/+}$ genotypes) aged 45 weeks; n=7-8 (G). Results are shown as mean ± SEM. Significance is indicated by *for p<0.05, ** for p<0.01, *** for p<0.00 between *ad libitum* and CR animals. Gene expression data was analyzed by multiple t-tests between the *ad libitum* fed group and the CR group within each adipose depot, with p<0.05 as considered significant.

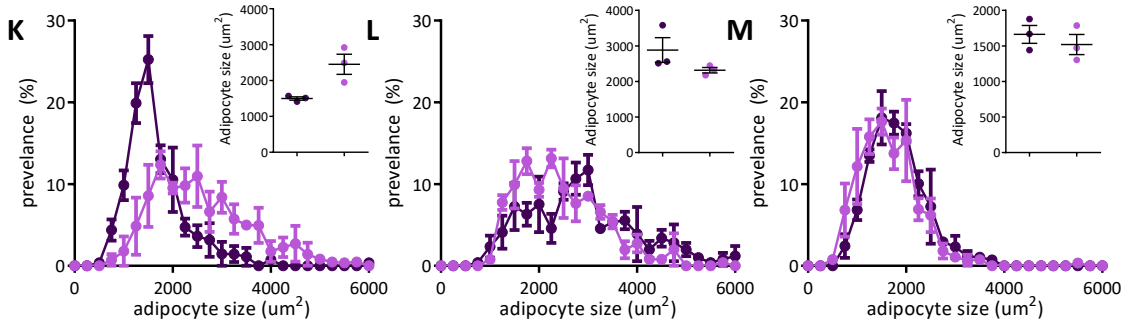
Supplementary table1: **Sybr Green PCR primer sequence**; UBC Vancouver, Canada

Supplementary figure 1: **CR-induced metabolic improvements are independent of insulin dosage in female *Ins2*$^{-/-}$ mice.** CR vs. ad libitum feeding, started at different ages, didn't affect *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ and *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ female mice differently based on body weight (A), pancreatic islets insulin content (fed) (B), 4-hour fasting insulin levels (C), 4-hour fasting glucose levels (D), intraperitoneal glucose-stimulated (2g/kg) insulin secretion following 4-hour fast (E) intraperitoneal glucose (2g/kg) tolerance test following 4-hour fast (F) and insulin (0.75U/kg) tolerance test following 4-hour fast (G). Animals used; n=10-16 (20 weeks), n=18-20 (45 weeks) and n=4-16 (70 weeks). Results are shown as mean ± SEM. Significance is indicated by *for p<0.05, ** for p<0.01, *** for p<0.001, between ad lib and CR animals and ## for p<0.01 between *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ and *Ins1*$^{+/-}$:*Ins2*$^{-/-}$

Fig. 1



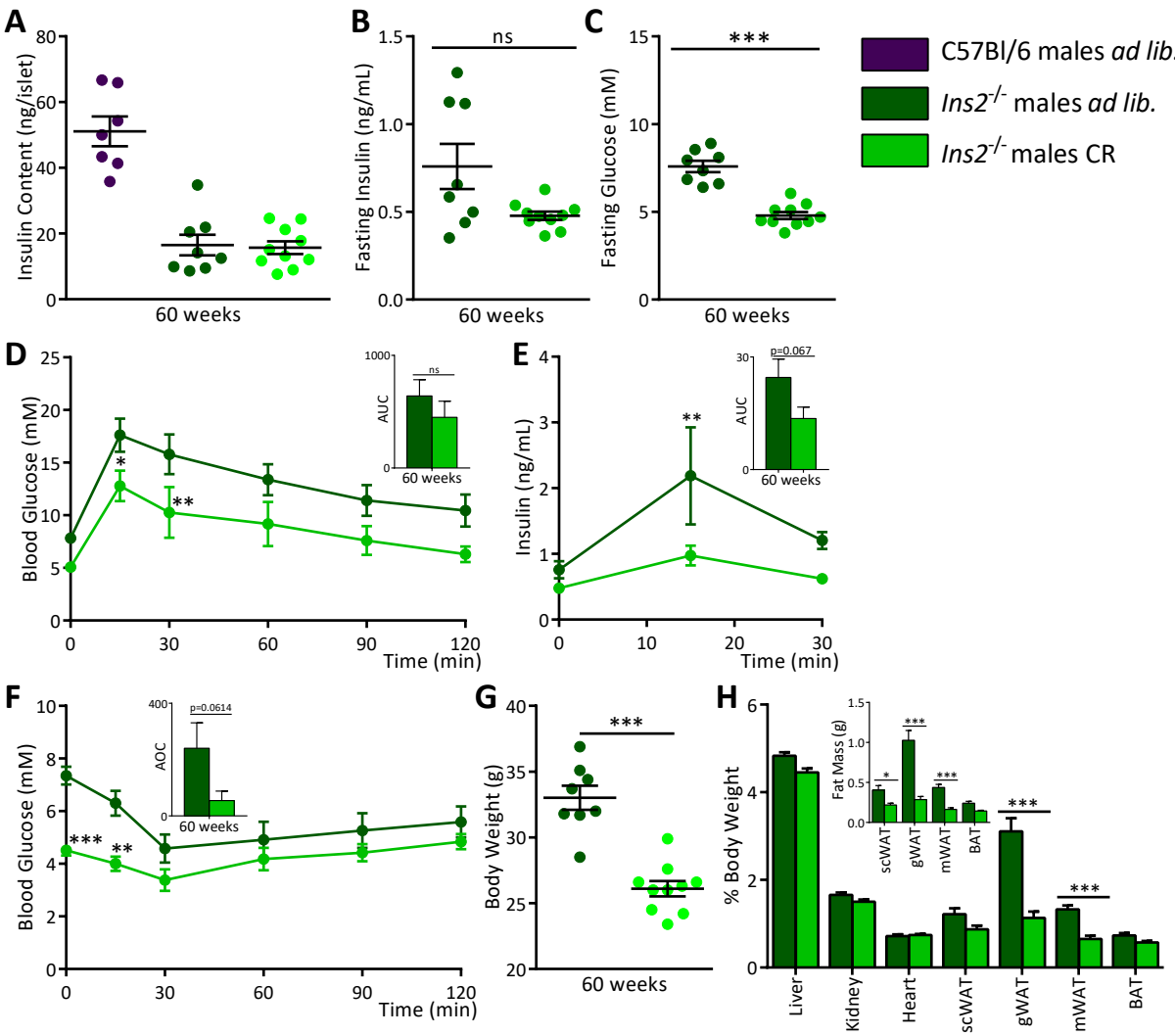




N

	gWAT			scWAT			mWAT			BAT		
	AL	CR		AL	CR		AL	CR		AL	CR	
Adrb3	100±21	78±27		100±8	98±11		100±10	78±8		100±15	68±14	
Cox4	100±19	115±17		100±11	143±12	*	100±6	118±9		100±13	69±17	
Prdm16	100±18	234±82		100±14	99±17		100±8	142±13	*	100±19	51±14	
Ucp1	100±53	48±10		100±51	22±4		100±18	84±12		100±14	49±9	*
Cebpb	100±23	99±18		100±12	94±14		100±10	85±10		100±17	49±10	*
Nrf1	100±36	175±54		100±11	105±13		100±8	111±9		100±21	39±6	
Pparg	100±24	124±27		100±11	149±13	*	100±20	124±19		100±13	56±9	*
Ppargc1a	100±23	132±16		100±12	136±18		100±11	181±28	*	100±18	30±8	*
Cidea	100±28	134±21		100±20	122±8		100±11	113±15		100±19	62±19	
Cideb	100±31	71±14		100±9	104±6		100±38	308±134		100±20	61±33	
Cidec	100±24	78±20		100±9	143±11	*	100±17	122±11		100±16	99±18	
Ddit3	100±15	125±21		100±10	142±12	*	100±7	105±8		100±21	54±13	
Acaca	100±30	166±37		100±17	153±12	*	100±13	120±17		100±17	39±15	*
Fas	100±29	131±31		100±17	155±14	*	100±12	114±13		100±16	44±15	*
Adgre1	100±21	89±20		100±11	87±5		100±8	83±6		100±26	84±51	
Insr	100±20	131±26		100±7	152±6	*	100±11	124±9		100±18	72±21	

Fig 2



I

	gWAT			scWAT			mWAT			BAT		
	AL	CR		AL	CR		AL	CR		AL	CR	
Adrb3	100±32	222±47		100±26	89±10		100±34	101±17		100±9	84±15	
Cox4	100±18	183±25	*	100±15	135±15		100±13	106±11		100±6	87±9	
Prdm16	100±25	52±6		100±14	86±10		100±18	97±9		100±10	64±5	*
Ucp1	100±40	55±14		100±40	99±20		100±19	44±6	*	100±6	53±3	*
Cebpb	100±29	109±19		100±29	60±7		100±12	91±8		100±15	87±8	
Nrf1	100±25	193±43		100±8	116±8		100±12	93±6		100±19	117±28	
Pparg	100±30	250±46	*	100±19	133±18		100±26	140±14		100±8	71±5	*
Ppargc1a	100±24	290±40	*	100±14	116±13		100±13	119±12		100±17	46±4	*
Cidea	100±23	520±81	*	100±31	186±26	*	100±14	160±13	*	100±5	108±8	
Cideb	100±36	228±42	*	100±11	110±10		100±52	58±33		100±13	68±8	*
Cidec	100±26	189±30	*	100±16	89±9		100±14	112±9		100±12	154±11	*
Ddit3	100±20	118±15		100±13	74±7		100±10	94±10		100±8	60±5	*
Acaca	100±25	517±76	*	100±14	170±17	*	100±23	154±12	*	100±4	73±4	*
Fas	100±30	378±48	*	100±16	189±18	*	100±20	156±12	*	100±5	75±4	*
Adgre1	100±19	162±21	*	100±18	88±9		100±10	96±10		100±21	85±13	
Insr	100±18	207±24	*	100±12	107±10		100±10	127±8		100±8	99±5	

Fig 3

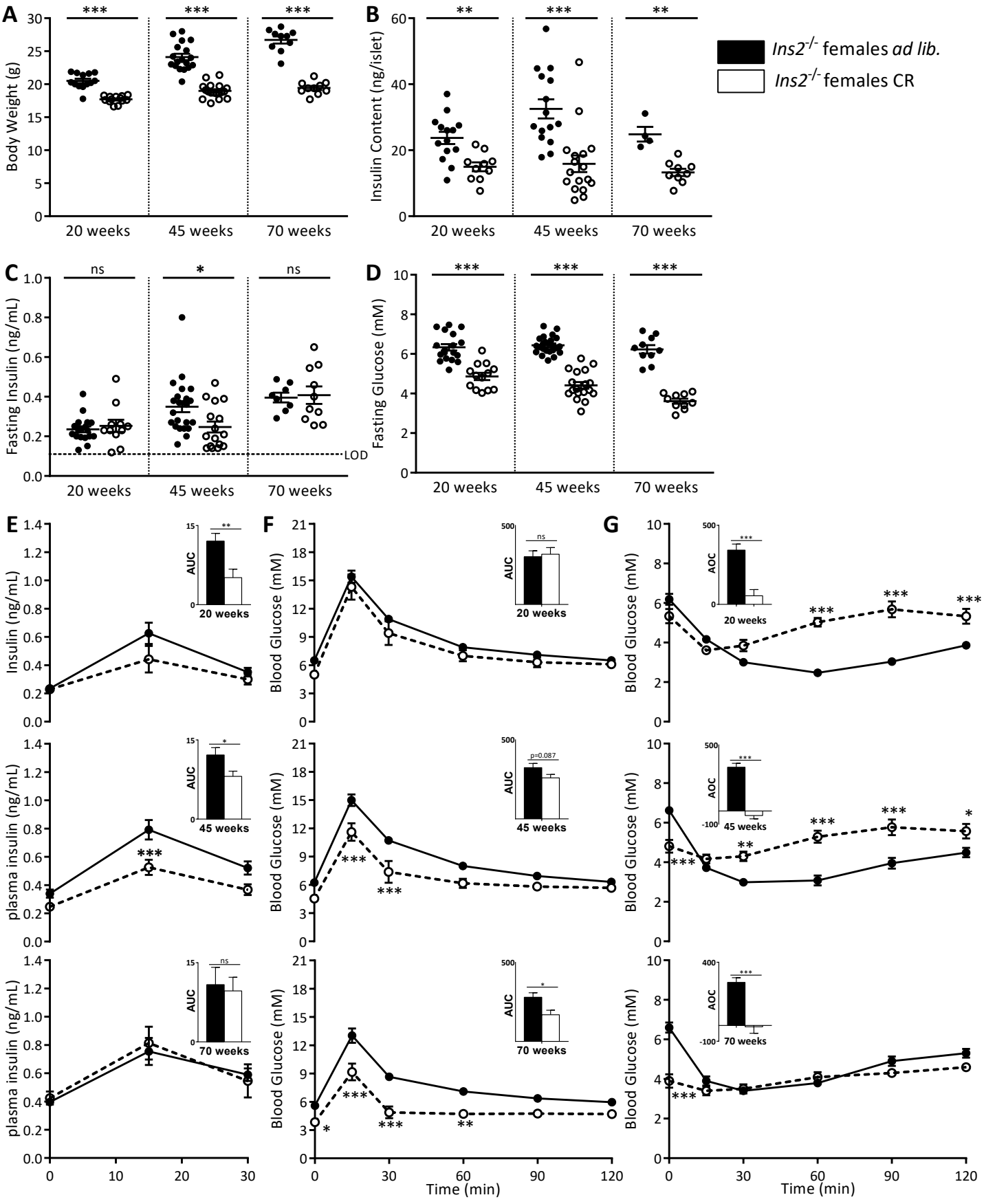
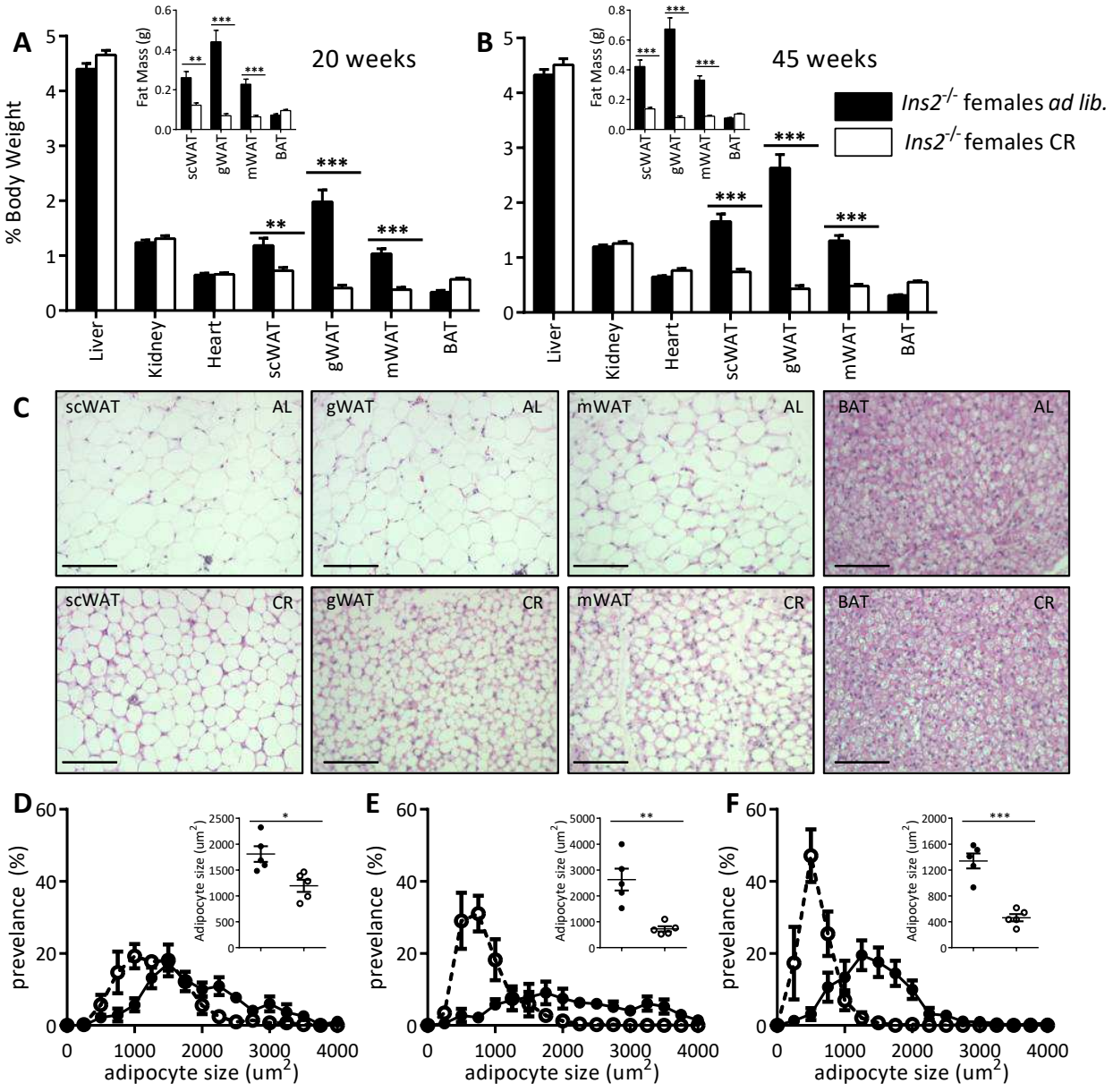

A
Body Weight (g)
20 weeks
45 weeks
70 weeks
B
Insulin Content (ng/islet)
C
Fasting Insulin (ng/mL)
LOD
D
Fasting Glucose (mM)
Ins2-/- females ad lib.
Ins2-/- females CR
E
Insulin (ng/mL)
plasma insulin (ng/mL)
AUC
F
Blood Glucose (mM)
G
AOC
Time (min)

Fig 4



G

	gWAT			scWAT			mWAT			BAT		
	AL	CR		AL	CR		AL	CR		AL	CR	
Adrb3	100±12	155±15	*	100±16	243±32	*	100±11	185±28	*	100±5	73±9	*
Cox4	100±4	108±10		100±8	225±29	*	100±11	152±18	*	100±5	143±12	*
Prdm16	100±5	173±21	*	100±9	233±12	*	100±16	175±32		100±6	171±24	*
Ucp1	100±19	12±4	*	100±35	553±293		100±22	137±67		100±8	110±10	
Cebpb	100±11	95±9		100±14	85±12		100±9	146±17	*	100±6	153±12	*
Nrf1	100±8	113±7		100±12	98±8		100±13	75±7		100±19	203±44	
Pparg	100±9	96±10		100±9	192±16	*	100±21	181±25	*	100±16	52±5	*
Ppargc1a	100±16	66±3	*	100±14	264±34	*	100±16	247±18	*	100±9	100±9	
Cidea	100±16	93±6		100±18	1034±114	*	100±19	391±100	*	100±7	153±14	*
Cideb	100±13	61±17		100±12	215±49	*	100±42	327±107		100±7	181±30	*
Cidec	100±6	121±7	*	100±10	210±20	*	100±23	128±16		100±6	225±31	*
Ddit3	100±7	109±9		100±4	166±13	*	100±3	161±6	*	100±9	139±12	*
Acaca	100±24	304±99		100±16	480±54	*	100±17	173±26	*	100±6	165±18	*
Fas	100±20	270±58	*	100±25	1479±279	*	100±25	174±26		100±5	147±16	*
Adgre1	100±8	67±6	*	100±10	50±4	*	100±13	66±4	*	100±14	118±16	
Insr	100±4	157±13	*	100±8	146±7	*	100±6	185±15	*	100±4	164±19	*